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The role and origin of pectin degrading enzymes during cassava retting

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- Abstract -

The origin of root softening during cassava retting was investigated.Two rettings in controlled conditions were performed. A "natural" retting in a bioreactor was used as a control and pH and dissolved oxygen were monitored. These conditions were then simultaneously imposed on a retting performed in sterile conditions. With this experimental set up, the role of pectic enzymes on the destruction of cellular walls evidenced. High pectin methyl esterase activities were assayed in cassava extracts and shown to be endogenous. Depolymerizing enzymes were also found, but only when microbial activity was present. A microbial lyase was found that depolymerized both methoxylated and non methoxylated substrates, with a slight preference for pectin. The pectinases involved in retting were then purified and partially characterized. Partially purified pectinemethylesterase had optimal pH and temperature of 7.0 an 30°C respectively, and the activity of the enzyme was stimulated by the presence of NaCl. The pectate lyase was inhibited by NaCl, but Ca2+ increased the specific activity. Optimal pH and temperature were 9.0 and 50°C respectively. The activity of these enzymes was further confirmed *in vitro* as they allowed the softening of cassava roots. However, the role of polygalacturonases was not clearly shown as their presence in retting was sporadic.

Therefore, root softening is due to the combined action of both endogenous pectin methyl esterase and exogenous depolymerizing enzymes - mainly lyase(s).

- Résumé -

L'origine du ramollissement au cours du rouissage a été étudiée à l'aide de deux rouissages effectués en conditions contrôlées. Le premier rouissage, dit "naturel", considéré comme rouissage témoin, est effectué en bioréacteur dans lequel le pH et la pression d'oxygène dissoute sont suivis en continu. Ces mêmes conditions (pH, T° et pO2) sont alors imposées à un rouissage effectué en conditions stériles. Ce dispositif expérimental a permis de déterminer le rôle des pectinases dans la destruction des cellules végétales. Des activités pectinemethylesterase ont été mesurées sur les racines fraîches et en cours de rouissage, cette activité est donc de nature endogène. Des enzymes dépolymérisantes (pectine lyase et polygalacturonase) ont été également mesurées mais seulement en présence de la flore bactérienne du rouissage. Une lyase d'origine bactérienne a été détectée qui dépolymérise mieux les pectines méthylées que les pectines non méthylées. Les pectinases du rouissage ont été alors purifiées et partiellement caractérisées. La pectinemethylesterase, partiellement purifiée, a un pH et une température optimum de respectivement 7 et 30°C. L'activité de l'enzyme est stimulée en présence de NaCl. En revanche, la pectate lyase est inhibée en présence de NaCl mais son activité spécifique est stimulée en présence de Ca2+. Le pH et la température optimale de cette enzyme sont respectivement de 9 et de 50°C. L'activité de ces enzymes dans le ramollissement de tranches de racines de manioc a été depuis confirmée par des essais *in vitro*. Cependant le rôle des polygalacturonases dans le ramollissement n'a pas été élucidé car leur présence dans le rouissage ne semble pas complétement établie. Le ramollissement des racines de manioc dans le rouissage est donc due à l'action combinée d'une pectinemethylesterase d'origine végétale et de pectinases bactériennes dépolymérisantes, principalement des pectines lyases.

Introduction

Retting, a spontaneous fermentation of cassava in Central Africa, is traditionally performed to soften the roots, degrade endogenous cyanogenic compounds and to give cassava-based foods their specific flavour. Microbiological, physico-chemical, and some biochemical aspects of this fermentation have already been extensively described (Brauman *et al.*, 1995 ; Okafor *et al.*, 1984 ; Oyewole, 1990), and the process has been optimized (Ampe *et al.*, 1994). However, the enzymatic mechanisms of root softening are not yet understood and the origin of detoxication is still controversal (Maduagwu, 1983 ; Okafor and Ejiofor, 1990 ; Ketiku *et al.*, 1978).

Degradation of plant cell wall can be due to the combined action of pectic enzymes as shown for flax (Chesson, 1980). This involves demethoxylating (pectinesterase) and depolymerizing enzymes (polygalacturonases and lyase). Pectate lyase (PAL) and polygalacturonase (PG) need deesterification before the degradation of natural pectic substances (e.g. those that cement plant cell walls) whereas pectin lyase (PNL) can degrade high methoxyl pectic substances. Pectin esterase (PE) are found in most vegetables but also - as inducible enzymes - in bacteria and fungi (Baterman and Millar, 1966). PG are mainly microbial, but are sometimes found in fruits and vegetables where they become active during maturation. PNL and PAL have only been described as microbial enzymes (Rexova-benkova and Marcovic, 1976).

Previous results have shown that cassava softening seems to be mediated by bacteria (Okafor *et al.*, 1984 ; Oyewole, 1990 ; Brauman *et al.*, 1995). Sterile roots soaked into sterile water did not ret, whereas microorganisms isolated from a previous retting could induce cell wall degradation. The presence of pectinesterase and lyase was only evidenced when cassava was inoculated with *Corynebacterium* spp. However, no pectolytic activity (and especially no pectinesterase) was found in fresh roots. More surprisingly, Okafor *et al.* (1984) found no pectolytic activity when inoculating with *Bacillus* sp. whereas retting could be completed. More recently, Oyewole and Odunfa (1992) have evidenced the presence of extracellular pectin methyl esterase during retting. However, these results do not allow any conclusion on the mechanisms of root softening, and the origin of these enzymes is not clearly understood yet.

Therefore, the present work attempts to define the role and origin of pectolytic enzymes in retting in order to elucidate the mechanism of root softening, with the scope of elaborating a bacterial starter to standardize this traditional fermentation. A "natural" fermentation used as a control, and a sterile one were performed simultaneously ; pH and oxygen pressure of sterile fermentation were

set on those of control fermentation during all the processing. Two pectinases involved in retting were then purified and partially characterized.

Materials and Methods

1. Origin of the plant material

Cassava roots (*Manihot esculenta* var. MM86 - "Ngansa") were harvested 18 months after planting, around the Brazzaville region in the Congo.

2. Fermentation conditions.

In order to determine the origin (vegetable, bacterial) of cell wall degradation and cassava detoxication, two fermentations were performed. A "natural" retting in a bioreactor (CF) was used as a control and to give indications on "natural" evolution of pH and dissolved oxygen. These conditions were simultaneously imposed onto a retting performed in sterile conditions. For both fermentations, 1.5 kg of peeled roots cut into cubes (1 cm3) were soaked in 2.5 litres of well-water in a 4.5 l bioreactors (SET 004M, Setric, Toulouse, France). Temperature was 32°C and agitation performed by medium recirculation (PPV peristalitic pump, SGI, Toulouse, France).

For sterile "fermentation" (SF), cassava roots cubes were sterilized with 0.1% HgCl2 in ethanol according to the procedure of Okafor et al. (1984). pH and partial oxygen pressure of the sterile fermentation were set on that of the control fermentation with the addition of 1 N HCl and nitrogen respectively.

Penetrometry index : Penetrometry was used as an indicator of roots softening during retting. A previous study has shown that a penetrometry index of 3 mm/s corresponds to the end of a retting as it is traditionally evaluated (Ampe et al., 1993). A Penetrometer (PNR 10 - SUR Berlin) was used to measure the consistency of the roots. 6 root sections were randomly chosen ; for each section, penetrometry depth was estimated with six repetitions.

Enzyme extracts. 80 ml of 0.1 M citrate buffer (pH 6.5) was added to 40 g of cassava mash and homogeneized in a Waring blender. The mixture was allowed to rest overnight at 4°C and the following day it was centrifuged at 20 000 rpm for 30 min. Supernatant was lyophilized and resuspended in 1/10 volume of citrate buffer.

3. Enzyme assays

Pectinemethylsterase (PE) activity was assayed by titration of 1 ml of enzyme extract in 1% pectin (Grindsted RS400 - DM 74%) containing 0.1 M NaCl and 1 mM NaN3 with 0,01 N NaOH at pH=7. One unit correponds to one µmol of NaOH/mn.

Pectate lyase (PAL) *and Pectin lyase activities* (PNL) were assayed following the procedures of Starr *et al.* (1977). with citrus Pectin (DM > 70%) for PNL and pectate for PAL.

Polygalacturonase (PG) was assayed by viscosimetry. 0.5 ml of enzyme extract was added to 40 ml of 1% pectin. Rate of reduction in viscosity was measured at pH 4 and at 25°C by a Haake VT 500 viscosimeter (rotation : 150.93 s-1 and system MV-MV1). One unit corresponds to one µmol of hexose released/mn.

Total activities are expressed as units per 100 g of cassava.

Cellulase and xylanase activities were assayed using the Somogyi-Nelson procedure (Nelson, 1944 and Somogyi, 1945). Substrates were respectively microcristallin cellulose (100 mg) and xylan (18 mg/ml). Temperature was 37°C and pH 5.8.

Protein content was evaluated using Lowry's procedure as modified by Bensadoun and Weinstein (1976).

4. Protein electrophoresis

For pectinase activities, PAGE was performed by incorporating pectin (Grindsted RS400 - DM 74 %) in acrylamide gels and subsequent staining with ruthenium red according to the Cruickshank and Wade (1980) method. Ruthenium-red stains polygalacturonic acid and the presence of polygalacturonase was shown by the presence of clear zones on the gel.

For ß-glucosidase activities, polyacrylamide gel was stained with 5mM PNPG in citrate buffer pH 6.5 for 1 hour. A positive yellow coloration was shown by alkalinisation with borate buffer pH 9.8.

5. Analytical methods

Action of pectic enzymes in vivo. Slices of cassava, sterilized as previously described, were inoculated with enzyme extracts and purified pectolytic enzymes purchased from SIGMA. 50µl of either enzyme extracts or 5µl of pure enzymes were adsorbed on root slices half-deeped in 0,01 M pH 5 citrate buffer. Softening of the plant material was estimated after 24 and 48 hours at 30°C.

6. Enzyme Partial Purification

Crude extracts were precipitated with 80 % ammonium sulfate, dialysed overnight again a tris HCL 20 mM buffer, pH 8.5 and concentrated. 20 ml samples were applied on DEAE Sepharose Fast flow column (20 ml) equilibrated with the same buffer. Evolution was performed by a NaCl gradient ; 2.5 ml fractions were collected for analysis. The active fractions were further purified with Q-Sepharose.

Results

1. The Origin of Softening

In control fermentation (CF), retting was completed in two days, but no softening was evidenced in sterile fermentation (SF) (Fig. 1). Despite the physicochemical changes imposed on SF (pH drop, anaerobiosis), cell walls were not degraded as shown by microscopic observation.

Significant PE activity was found in cassava fresh roots (465 U/100 g of cassava dry matter. Figure 2a). Total activity in CF and SF decreased all along the process. Activity in CF was slightly higher than in SF.

Depolymerizing enzymes were found in CF from 20 hours onto the end of fermentation, but not in SF. Total polygalacturonase activity in control fermentation ranged from 92 to 135 U/100 g cassava dry matter (Figure 2b). Non denaturing electrophoresis of enzyme extracts followed by specific staining with ruthenium red confirmed the presence of polygalacturonase.

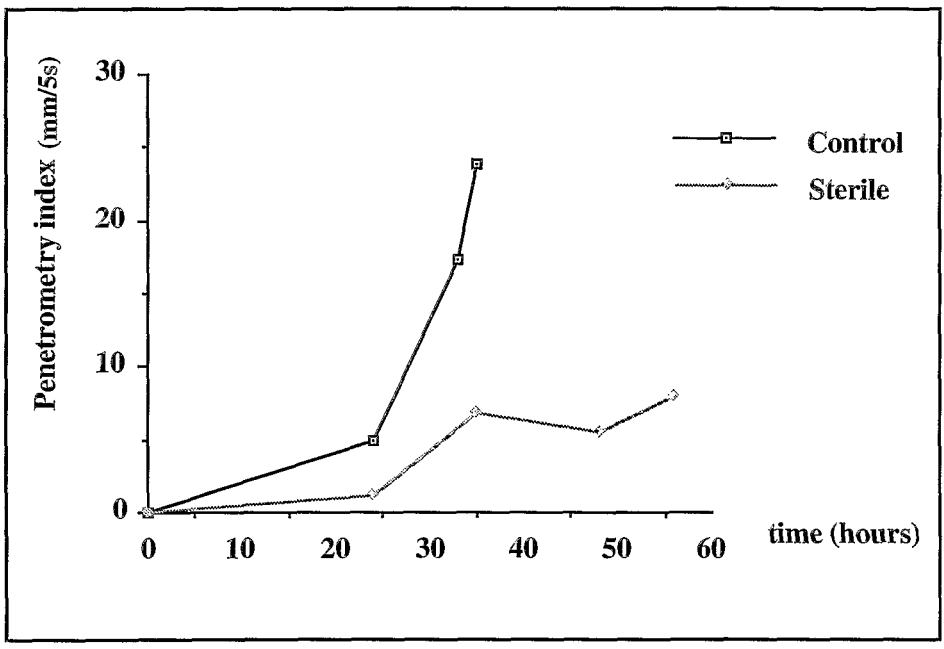

Figure 1
Evolution of pnetrometry index of both Control and Sterile Fermentation

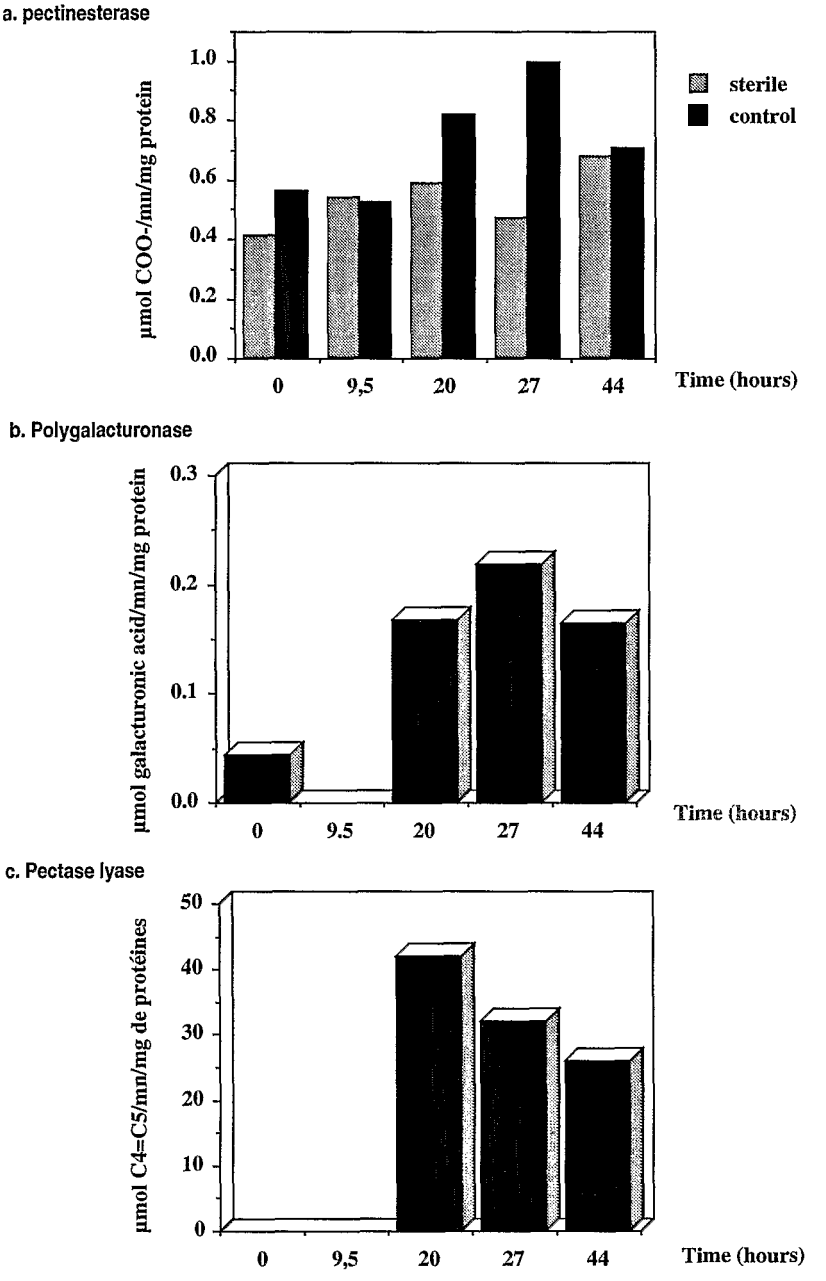

Figure 2

Pectinases activities in sterile and control retting. a : pectinesterase ; b : polygalacturonase ; c : pectate lyase. Black boxes stand for control retting and grey ones for sterile one

Clear zones are very intense for samples from 20 hrs to the end of CF whereas gel was not decolorated for samples from sterile retting and with fresh cassava. Decoloration of ruthenium red was very intense showing a high activity on pectin compared to that of purified enzymes. Distance of migration was the same for all positive samples, and very low compared to the control enzyme from *A. niger*.

PAL activity (79 to 184 U/100 g of cassava dry matter) was found in CF after 20 hours of fermentation and stayed stable until the end of the process (Figure 2c). No activity was detected in SF. The presence of PNL, not present in this experiment is confirmed ; PNL are present during retting, but the activities assayed in this study were very low at acid pH (unpublished results).

In vivo (Figure 3) Pectolytic activity on cassava cells was confirmed by inoculating fresh sterile cassava with commercial enzymes. After 24 hours at 30°C, tissues inoculated with PE+PG, PE+PAL and PE+PAL+PG were almost totally degraded. No other depolymerizing enzyme as xylanase or cellulase were found in either retting.

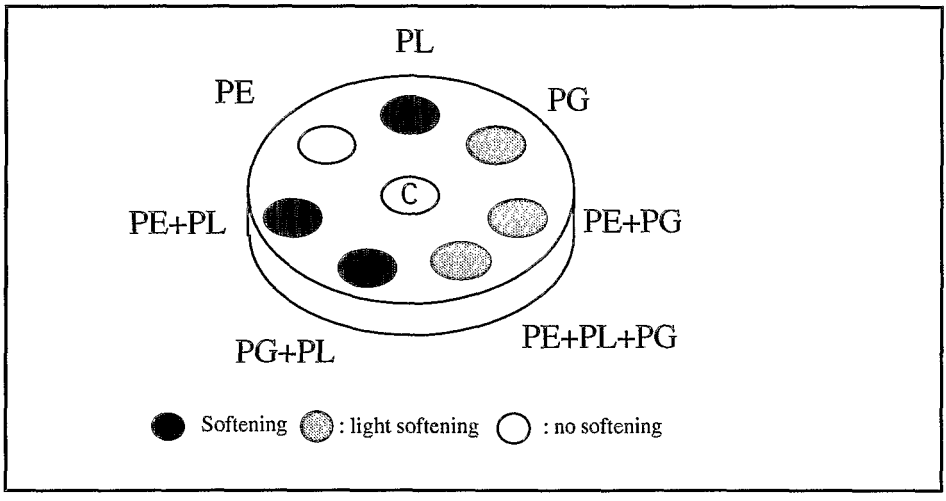

Figure 3
Activity of pectic enzymes on fresh cassava slices

2. First Steps towards Purification of a Pectinesterase and a Lyase

Cassava pectinesterase and a depolymerizing fraction characterized as pectin lyase were partially purified using ion exchange chromatography with NaCl as gradient. Purification yields were 17.45 and 9.83 respectively and final specific activities reached 29.68 and 2.90 units/mg protein respectively (Table 1).

Table 1

Purification of pectinesterase (1.a) and Pectin lyase (1.b) during cassava retting. Legends: Total Act.: Total Activity; S.A.: Specific Activity; Purif.: Purification; Activities are given in ml of NaOH/mn/0.2 ml of extract for pectineesterase, U.O.D/mn/0.2 ml of extract for Pectin lyase, volume in ml and specific activity (S.A.) in units/mg protein

a: Pectinesterase

Sample	Activity	Volume	Total Act.	S.A.	Purif. Factor	Yield
Crude extract	0.019	100	9.5	1.70	1.0	100 %
$(NH_4)2SO_4$	0.43	3	6.45	2.26	1.33	68 %
conc. & dialysed	0.1575	4	3.15	2.38	1.40	33 %
DEAE Sepharose	0.095	2.5	1.19	29.7	17.45	13 %

b: Pectin lyase

Sample	Activity	Volume	Total Act.	S.A.	Purif. Factor	Yield
Crude extract	0.0033	100	1.66	0.295	1.0	100 %
$(NH_4)2SO_4$	0.057	3	0.86	0.30	1.02	52 %
conc. & dialysed	0.0213	4.5	0.48	0.32	1.08	29 %
DEAE Sepharose	0.0093	2.5	0.12	2.9	9.85	7 %

3. First Characterization of Retting Pectinases

Pectinesterase and pectin lyase identified during retting were partially characterized. The activities are shown in Figures 4 and 5.

Pectinesterase has an optimal pH at 7, but was still active in the retting pH range of 4.5-6. On the other hand, alkaline pH were best for pectin lyase, and the enzyme kept only 10 % of its maximal activity for pH values below 6.

Temperature profiles were found to be quite different in this study. The optimal temperature for pectinesterase activity was consistent with that of processing (around 30°C), whereas pectin lyase was more active at a higher temperature (around 50°C).

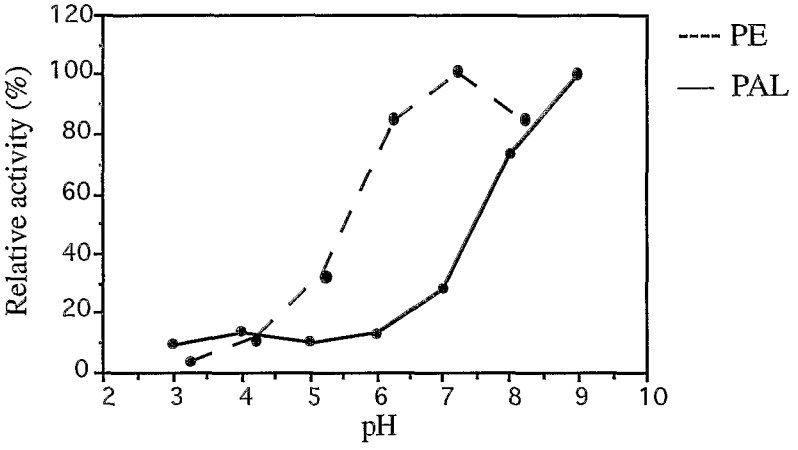

Figure 4
Relative activity of pectinesterase and pectin lyase as a function of pH.

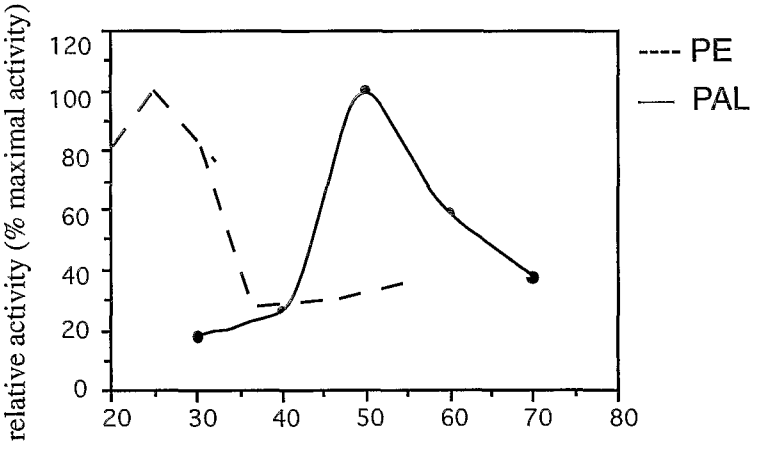

Figure 5
Relative activity of pectinesterase and pectin lyase as a function of pH.

Besides, the role of salts on lyase activitiy was investigated. Calcium chloride increased enzyme activity by two-fold, whereas EDTA had almost no effect and NaCl (100 mM) decreased the activity by 75 %. Lyase substrate specificity was also tested (Figure 6). Methylated pectin was degraded more than polygalacturonic acid; all pectins tested could be used for this broad substrate enzyme.

Discussion

From these results, it can be assessed that softening is not due to a chemical stress, and that microbial enzymes are indispensible for retting to be completed.

The high activity of PE in fresh cassava confirms its plant origin. It is bound to cell wall intracellular space by high ionic strengths (Versteeg, 1979), and might be released by the soaking of roots in water.

On the other hand, depolymerizing enzymes (PG, PA or PNL) were not detected in fresh roots and SF. In CF, significant enzyme activities were assayed after 20 h of fermentation, as the microbial population was already fully established (Brauman et al., 1995), thus indicating their microbial origin. PG optimal activity was compatible with the low pH of the fermentation (4 to 5). PAL was also active at pH 5 but retained only 10% of its maximum activity (optimum pH 9.0).

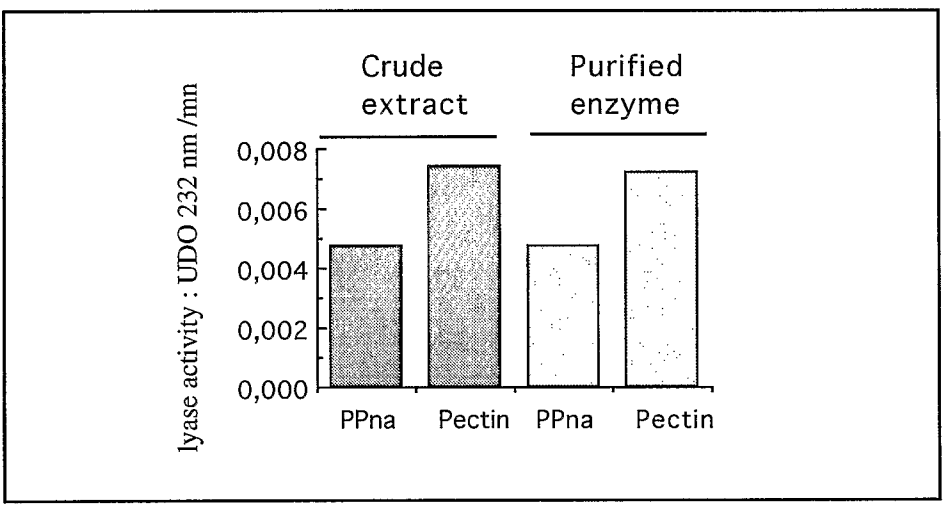

Figure 6

Pectin lyase activity on pure and crude extract substrates. Dark boxes stand for crude extract and clear ones for purified enzyme.
Legends: ppNa: Non Methylated pectine
Pectin: Methylated Pectin (75%)

These results suggest that cell wall degradation is due to pectolytic enzymes from both plant and bacterial origin. It should be noted that the absence of lyase activity in SF corroborates results from other studies that pectate lyases are only from microbial origin (Fogarty and Ward, 1974).

The combined actions of pectinesterase, polygalacturonase and/or lyase was confirmed by inoculating pure enzymes on fresh cassava roots. Cassava softening can therefore be compared to cucumber or flax cells degradation (CHESSON, 1980). It could be suggested that the destruction of pectic chains starts with demethoxylation by PE followed by the action of depolymerizing enzymes; polygalacturonase and/or lyase, the former being the more active at low pH.

Various bacteria are able to produce pectinases, some lactic acid bacteria such as *L.plantarum* and *L.mesenteroides* - two main bacteria of the cassava retting (Malonga *et al* ,1995)- possess polygalacturonase or pectate lyase activities (Sakellaris et al., 1989 ; Juven et al., 1985). But other bacteria involved in the process such as *clostridia* could be responsible for pectin degradation, as previously described for potatoes softening (Lund, 1972). Lactic bacteria, as well as other bacteria isolated from previous rettings, will be further screened for polygalacturonase and lyase activities in order to select strains for the elaboration of a bacterial starter. At the same time, the enzymes will be purified from retted mash, and their mode of action characterized.

This work supports the first evidence for the presence of PG in retting. PE was found by Oyewole and Odunfa (1992) but its plant origin was not demonstrated. Besides, the only description of lyase activity in retting was made after the inoculation of cassava with *Corynebacterium spp.* (Okafor *et al.*, 1984), and its role in retting remains unclear.

The specific action of purified lyase has to be confirmed, especially as a function of pH and the mode of action (endo or exo) also has to be defined.

Conclusion

Retting is a spontaneous fermentation in which endogenous and microbial enzymes co-act to soften the roots. The results obtained in this study suggest that cell wall degradation is initiated by endogenous pectin esterase located in intercellular space and released by pH decrease, followed by the action of microbial polygalacturonase and pectate lyase that depolymerize pectic chains. Pectin lyase could also act directly on methylated pectins.

These results provide precious informations for the elaboration of bacterial starter. Experiments are in progress for the characterization of pectinolytic microorganisms with ecological relevance to retting.

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